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11 November 1995

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REQUEST FOR GRANT OF A PATENT

PA 22238

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THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION

I Applicant's or Agent's Reference *(Please insert if available)* PA87

II Title of Invention CHIMERIC PROTEINS

P

III Applicant or Applicants *(See note 2)*

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~~or~~
~~(b) A statement on Patents Form No. 7/77 is/will be furnished~~

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~~VII Declaration of Priority (See note 6)~~

~~Country~~ ~~Filing date~~ ~~File number~~

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~~VIII The Application claims an earlier date under Section 8(3), 12(6), 15(4), or 37(4) (See note 7)~~

~~Earlier application number~~ .... ~~and filing date~~ ....

V4811

IX Check List *(To be filled in by applicant or agent)*

A The application contains the following number of sheet(s)			B The application as filed is accompanied by:-	
1	Request 1	Sheet(s)	1	Priority document
2	Description 18	Sheet(s)	2	Translation of priority document
3	Claim(s) —	Sheet(s)	3	Request for Search
4	Drawing(s) 6	Sheet(s)	4	Statement of Inventorship and Right to Grant
5	Abstract —	Sheet(s)		

X It is suggested that Figure No 1 of the drawings (if any) should accompany the abstract when published.

XI Signature *(See note 8)* Patricia E Crawley Chartered Patent Agent Agent for the Applicant

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REF: PA87

CHIMERIC PROTEIN

This invention relates to chimeric proteins and to processes for their production using recombinant DNA techniques. For the purposes of the present description the term 'chimeric protein' means a protein comprising the whole or part of the amino acid sequence of at least two separate proteins. In particular the invention relates to chimeric proteins in which one of the at least two separate proteins comprises the whole or part of the heavy and light chain variable regions of an immunoglobulin molecule, and which has functional antigen binding activity.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of a wide range of heterologous polypeptides and proteins in host cells which have been transformed with heterologous DNA sequences which code for the production of these products. Thus European Patent Application EP 0088994 (Schering Corporation) proposes the construction of recombinant DNA vectors which carry a ds DNA sequence that codes for a variable region of a light or heavy chain of an immunoglobulin specific for a predetermined ligand but lacks nucleotides coding for aminoacid residues superfluous to said variable region and is equipped for initiation and termination codons at the 5'- and 3'-termini respectively of said DNA sequence. It is proposed that these vectors can be used to transform host organisms, in particular bacteria, to produce recombinant DNA molecules consisting of the whole or part of the heavy and light chain variable regions of selected

immunoglobulins. Furthermore, European Patent Application EP 1 102 634 (Takeda Chemical Industries Limited) describes the cloning and expression, in bacterial host organisms, of genes coding for the whole or part of a human immunoglobulin E heavy chain polypeptide.

We have now succeeded in producing, using recombinant DNA techniques, chimeric proteins comprising heavy and light chain variable regions of an immunoglobulin molecule together with a second polypeptide sequence derived from a different source. Surprisingly we have found that these chimeric proteins possess both the functional antibody binding activity of the heavy and light chain variable regions and also the functional activity of the second polypeptide sequence derived from the different source.

Accordingly the invention provides, as a product of recombinant DNA technology, a chimeric protein comprising heavy and light chain variable region amino acid sequence of a defined immunoglobulin together with at least one further polypeptide derived from a different source.

Characteristically the further polypeptide(s) is heterologous (foreign) to the organism used for expression of the chimeric protein.

Typically the chimeric proteins of the invention possess functional antibody binding activity provided by the heavy and light chain variable region sequence. This antibody binding activity may comprise any appropriate antibody/antigen (including hapten) binding activity. For instance the chimeric protein may bind to a cell surface antigen or hapten such as a hormone. We describe chimeric proteins which bind to a nitrophenyl (NP) hapten though it will be appreciated that the invention is widely applicable to antibody binding specificities in general. The heavy and light chain variable region sequence may be derived from a monoclonal antibody.

Typically also the chimeric proteins of the invention, in addition to the functional antibody binding activity, possess further functional activity or activities provided by the at least one further polypeptide. The further polypeptide(s) may comprise immunoglobulin sequence derived from a different immunoglobulin molecule. The further polypeptide may comprise constant region immunoglobulin sequence including human constant region immunoglobulin sequence e.g. a human epsilon heavy chain region sequence.

Alternatively the further polypeptide(s) may comprise a non-immunoglobulin polypeptide, such as a drug, cytotoxic agent or enzyme. For example, the further polypeptide may comprise an enzyme such as a nuclease.

Without prejudice it is believed that chimeric proteins according to the invention will be useful for therapy and diagnosis. For instance, when the chimeric protein has antibody binding activity for a cell surface antigen, such as a cancer cell surface antigen, and the further polypeptide (s) comprises a cytotoxic agent the chimeric protein may be used as a selective cytotoxic agent e.g. to selectively kill cancer cells. Also, for example, chimeric proteins in which the further polypeptide(s) comprises an enzyme may be used as enzyme labelled immunoassay reagents.

Advantagously also the chimeric proteins of the invention may provide methods for obtaining the further polypeptides in very pure state. Thus crude product comprising the chimeric protein may be immunopurified using the functional antibody binding activity. For example crude chimeric protein product is contacted with solid phase comprising the antigen/hapten to which the variable region sequence binds, the solid is separated from the crude mixture, washed and the purified chimeric protein recovered from the solid phase. The further polypeptide may be recovered from the chimeric protein by appropriate cleavage if desired.

The chimeric proteins of the invention may be produced by methods analogous to those which are already known and used in the recombinant DNA art. Thus hybrid genes, comprising DNA coding for at least part of the variable region of an immunoglobulin chain together with DNA coding for the further polypeptide may be constructed and these hybrid genes used to transform/transfect competent host organisms. Preferably a gene for at least part of a heavy chain variable region is joined to a gene coding for the further polypeptide to provide a hybrid gene.

The hybrid genes so produced may be used to transform/transfect host cells which already produce the corresponding immunoglobulin light or heavy chain e.g. a myeloma or a hybridoma line or a line derived therefrom. Alternatively host cells may be transformed with genes coding for both light and heavy chains, and both such genes may be hybrid genes each comprising DNA coding for further polypeptide(s).

Any suitable host organisms may be used for transformation/transfection, though eucaryotic host organisms, especially mammalian cell lines, such as myeloma lines, are preferred. In accordance with the present invention we have discovered that the chimeric proteins are produced in good yield in eucaryotic host cells and are advantageously secreted from the host cells into the culture supernatant.

The invention also includes processes for the production of chimeric proteins and methods for their use in therapy, diagnosis and purification (of the further polypeptide) as outlined above.

The invention is further described by way of illustration only in the following examples.

EXAMPLE 1

A cell line has been developed which secretes an immunoglobulin molecule with a mouse anti-NP variable region and a human epsilon heavy chain constant region. A recombinant DNA clone was constructed with vector PSV2 GPT containing the mouse VH (anti-NP) and human CE segments (Flanagan & Rabbits, EMBO J. Vol. 1, No. 5, pp. 655-660, 1982). This clone was transfected into the established mouse myeloma cell line J558L (which makes only alpha light chains). After stable integration of the recombinant clone into myeloma cells, a cell clone (JW8/5/13) was established which secretes immunoglobulin (heavy and light chains) easily purified on NP-affinity columns. This protein contains the whole human CE portion and can be used as a competitor in radio-immunoassay for quantitation of human IGE. The CE portion of IGE is the part which normally binds to mastcells during allergic responses. In collaboration with Dr. B. Mitchell (CRC, Northwick Park Harrow) we are currently testing the use of this recombinant protein for competitive <u>in vitro</u> and <u>in vivo</u> blocking of mast-cell receptors.

CHIMETRIC PROTEINS

EXAMPLE TWO

Monoclonal antibodies secreted by hybrid myeloma cells[1] have not only found widespread use as diagnostic reagents[2] but are also being employed in therapy.[3] The recent emergence of techniques allowing the stable introduction of immunoglobulin gene DNA into myeloma cells[4-6] has opened up the possibility of using in vitro mutagenesis and DNA transfection to construct recombinant antibodies possessing novel properties. In particular, one can imagine that the production of chimeric proteins in which the antigen binding portion of the immunoglobulin is fused to an enzymic moiety could prove of use in immunoassays. Furthermore, the chemical coupling of toxins to cell specific antibodies has been advocated as a technique in chemotherapy[7] and antibody fusions containing cytotoxic carboxyterminal moieties could be exploited in this area.

Myeloma cells appear to provide an attractive system for the production of chimeric antibodies as these cells are specialised in the synthesis and secretion of large amounts of immunoglobulin. However, at present, we neither know whether chimeric antibodies can be secreted by a myeloma cell nor do we know whether an antibody/enzyme fusion will retain both antigen binding and enzymatic activity. In this work, we describe experiments which test the feasibility of making recombinant antibodies in myeloma cells.

Reconstitution of hapten specific antibody

We have previously described[5] the construction of plasmid pSV-V_H1 which includes a complete mouse immunoglobulin μ gene cloned in the expression vector pSV2gpt (Fig. 1). The immunoglobulin μ polypeptide encoded by this plasmid has a heavy chain variable region, V_H, characteristic of λ_1 light

chain-bearing mouse antibodies that are specific for the hapten 4-hydroxy-3-nitrophenacetyl (NP); NP-binding activity should therefore be formed following association of the pSV-Vμ1 encoded heavy chain with mouse λ_1 light chains. To confirm this, pSV-Vμ1 DNA was introduced by spheroplast fusion into J558L, which is a mouse plasmacytoma that expresses λ_1 but produces no heavy chain[4]. Stably transfected cells were selected as described in the legend to Fig. 2 and were cloned by limiting dilution. Twenty clones were analyzed by radioimmunoassay and each secreted high levels of an NP-specific IgM antibody (data not shown). As illustrated in Figure 2, homogeneous anti-NP IgM antibody can be purified from supernatants of pSV-Vμ1 transfected clones with a yield of about 3 mg/l. Note that the purification is a simple one step procedure performed using a hapten-Sepharose column.

Secretion of F(ab)'-like antibody

To test whether deletion of the Fc portion of the heavy chain gene results in impaired antibody synthesis or secretion, a derivative of pSV-Vμ1 was constructed in which the Cμ exons were replaced by the CH1 and hinge exons of the mouse γ2b gene. To provide translation termination and polyadenylation sequences, an exon, $C\delta_s$, derived from the gene encoding secreted mouse δ chains was placed at the 3' end of the gene (Fig. 1). The truncated heavy chain gene of this plasmid pSV-$V_{NP}\gamma\delta$ would be expected to direct the synthesis of a $F(ab)'_2$ like molecule consisting of two IgG2b Fab molecules disulphide linked together through the γ2b hinge and with a 21 aminoacid tailpiece at the carboxy terminus encoded by the $C\delta_s$ exon. Plasmid pSV-$V_{NP}\gamma\delta$ was transfected into J558L cells and radioimmunoassay revealed that the stably transfected cells secreted high levels of λ_1-bearing anti-NP antibody. This NP-specific antibody was purified from culture supernatants of several transfected clones with a yield of 5 to 10

mg/l. Polyacrylamide gel electrophoresis of the purified material (Fig. 2c) shows that the major protein species has an unreduced molecular weight of about 110,000 daltons. After reduction, a band comigrating with λ light chain as well as several higher molecular weight polypeptides are observed. The most abundant of these larger polypeptides has a molecular weight of 31,000 and would constitue the heavy chain of the $F(ab)'_2$ like antibody. However, there is clear contamination of the pSV-$V_{NP}\gamma\delta$ $F(ab)'_2$ antibody with other NP-binding material that has an unreduced molecular weight of around 50,000 daltons and is composed of λ_1 light chains and one of several heavy chains in the molecular weight range 36,000 to 40,000. The presence of this minor antibody component does not reflect glycosylation heterogeneity as the electrophoretic mobility of the pSV-$V_{NP}\gamma\delta$ encoded anti-NP antibody is unaffected by inclusion of tunicamycin in the incubation medium during biosynthetic labelling experiments (Fig. 2b). It is likely that the minor bands differ from the $F(ab)'_2$ antibody in the carboxy terminal portion of the heavy chain, possibly as a result of alternative processing of pSV-$V_{NP}\gamma\delta$ immunoglobulin gene RNA transcripts. Nevertheless, despite the contaminating bands, it is clear that $F(ab)'_2$ like anti-NP antibody can be synthesized and secreted in good yield by pSV-$V_{NP}\gamma\delta$ transfected J558L cells. This is in keeping with the observations of Sharon et al[8] who found that monovalent recombinant Fab-like molecules could be secreted by transfected myeloma cells.

Secretion of Fab-nuclease

As a novel activity to fuse to the antibody heavy chain we chose the nuclease from Staphylococcus aureus. We selected this enzyme as it is a secreted enzyme which is active as a monomer, contains no thiol residues and can easily refold after denaturation[9]. A DNA restriction fragment containing the S. aureus nuclease (SNase) gene was inserted into the XhoI

site located in the CH2 exon of the mouse γ2b gene. Plasmid pSV-V_{NP}γSNase was assembled as described in the legend to Fig. 1; the heavy chain gene of pSV-V_{NP}γSNase is similar to that of pSV-V_{NP}γδ except that the $C\delta_s$ exon has been removed and replaced by an exon containing the first four codons of the γ2b CH2 exon fused in phase to the nuclease coding region. SV40-derived sequences of the pSV2gpt-derived vector provide polyadenylation signals. J558L cells were transfected with pSV-V_{NP}γSNase and cells surviving in selective medium were cloned by limiting dilution. Radioimmunoassay of supernatants of cloned transfectants revealed that about one third were positive for the production of λ-bearing anti-NP antibody. Positive clones yielded between 1 mg/l and 10 mg/l of NP binding antibody. Analysis of biosynthetically labelled antibody by gel electrophoresis reveals a band comigrating with λ_1 light chain as well as two heavy chain bands of molecular weight 45,000 and 46,000 (Fig. 2b). The difference between these two heavy chains has not been identified but their mobilities agree well with the predicted mobility of the V_{NP}γSNase heavy chain. Although the sequence Asn-Asn-Thr is present in SNase, the two V_{NP}γSNase heavy chain bands are still present in samples purified from supernatants of cells that have been biosynthetically labelled in the presence of tunicamycin (Fig. 2). This demonstrates that the difference between the two pSV-V_{NP}γSNase heavy chains is not due to N-linked glycosylation. The V_{NP}γSNase antibody appears somewhat more heterogenous on a non-reduced gel, giving bands with the expected mobilities of both the $F(ab)'_2$-SNase and Fab-SNase. The presence of SNase on the heavy chain carboxy terminus might inhibit disulphide linking of the γ2b hinge regions.

Fab-nuclease is enzymatically active

To test for nuclease activity in the V_{NP}γSNase preparation, samples which had been purified on hapten-Sepharose columns were incubated with single

stranded DNA substrate. Digestion of the DNA was monitored following agarose gel electrophoresis. As shown in Fig.3, $V_{NP}\gamma$SNase but not $V_{NP}\gamma\delta$ antibodies show nuclease activity and this activity - like that of authentic S. aureus nuclease - is dependent on Ca^{++} but not Mg^{++} ions. As judged on a molar basis, the catalytic activity of the $V_{NP}\gamma$SNase sample is about 10% that of authentic S. aureus nuclease.

Fab-nuclease can be used in immunoassay

The $V_{NP}\gamma$SNase antibody can be used as a genetically conjugated enzyme linked antibody in ELISA-type assays. Antigen-coated plastic plates were incubated with various amounts of $V_{NP}\gamma$SNase protein and bound antibody was then detected by virtue of its nuclease activity. This was achieved by addition to the plate of a solution containing DNA and ethidium bromide. Following digestion of the DNA substrate by the immoblized $V_{NP}\gamma$SNase antibody, the fluorescence due to to the DNA/ethidium bromide complex substantially decreased. As shown in Fig. 4, quantities in the range of 10 ng of $V_{NP}\gamma$SNase antibody are easily detected and no decrease in fluorescence is obtained with the $V_{NP}\gamma\delta$ antibody control. We have found that the assay may be made at least tenfold more sensitive by increasing the incubation time with the DNA substrate.

Secretion of Fab-myc

As discussed above, the nuclease is an attractive moiety to fuse to an antibody due to its stability, ease of renaturation and lack of thiol groups and the fact that it is itself a secreted protein... We therefore decided to fuse the carboxyterminal portion of mouse c-myc to the antibody Fab in order to test the generality of the approach. The product of the c-myc gene is a protein which contains many thiol groups and is normally resident within the cell. There is no reason to believe that the third

exon of c-myc on its own will encode a functional protein domain. Thus, if the Fab-myc fusion protein were secreted from the cell, not only would it provide a source of protein for making anti-myc antisera, but it would also allow us to assess the general feasibility of secreting chimeric antibodies. Plasmid pSV-$V_{NP}\gamma$myc was assembled as described in the legend to Fig. 5; the plasmid is similar in structure to pSV-$V_{NP}\gamma\delta$ except that the $C\delta_g$ exon is replaced with the 3'-terminal exon of the mouse c-myc gene. This c-myc exon encodes 187 aminoacids[10] and should provide the transcription polyadenylation signal. The plasmid was transfected into J558L and cells from wells positive for production of anti-NP antibody cloned by limiting dilution. Hapten-binding protein was purified from culture supernatants and analyzed for the presence of c-myc antigenic determinants in an indirect radioimmunoassay. Samples of either the putative Fab-myc or of the anti-NP $F(ab)'_2$ (to act as control) were incubated in wells of a polyvinyl microtitre plate that had been coated with a monoclonal anti-c-myc antibody; bound anti-NP antibody was then detected using a radioiodinated monoclonal anti-idiotope antibody which recognizes the Fab portion of the anti-NP antibodies. As shown in Fig. 6, the Fab-myc clearly binds to the monoclonal anti-c-myc antibody, whereas the anti-NP $F(ab)'_2$ and other controls do not. The Fab-myc was also recognized by two other monoclonal antibodies that are specific for the carboxyterminal end of c-myc (data not shown). SDS/polyacrylamide gel electrophoresis (Fig. 5) of the Fab-myc reveals that it is somewhat heterogenous; a banding comigrating with λ_1 light chains and several bands with higher molecular weight in the range 38,000 to 55,000 daltons are observed, without a single dominant heavy chain band being apparent. The expected size of the Fab-myc is 50,000 daltons. We have observed that, after prolonged storage, precipitates appear in the Fab-myc sample and SDS/polyacrylamide gel electrophoresis reveals more extensive heterogeneity

of the heavy chain bands. We therefore believe that the heterogeneity of the Fab-myc protein indicated by the SDS/polyacrylamide gel analysis is most probably due to proteolytic degradation.

Conclusion

Myeloma cells can synthesize and secrete active recombinant antibodies. The Fab-nuclease is secreted in good yield, retains enzymic activity and is a stable protein. We have shown how such an antibody/enzyme fusion protein can be used in immunoassay and it is clear that analogous recombinant antibodies could find application in other fields. The results obtained with the Fab-myc construct suggest that whilst it may be possible to drive secretion of a large variety of different substitutions of the Fc portion, problems may be encountered with the stability of the resultant protein; in this case it is likely that the c-myc encoded part of the antibody is unable to fold as a protein domain. Nevertheless, the fact that c-myc antigenic determinants are clearly displayed by the Fab-myc protein suggests that this might be a valid approach to the preparation of antisera against the previously unidentified products of specific genes. Finally, it is worth noting that these antibody/enzyme fusions may provide an attractive means for driving the synthesis and extracellular secretion of specific enzymes as fused proteins which could then be facilely purified on antigen columns. The enzyme and antibody moieties could, if required, be dissociated using specific proteases[11].

We thank S. Morrison for the J558L myeloma, Gerard Evan for the monoclonal anti-c-myc antibody and Graham Hatfull, Franco Calabi and Cesar Milstein for lively encouragement.

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Figure Legends

Fig.. 1. a) Structure of plasmids. Thin horizontal lines depict the pSV2gpt vector, thick lines represent immunoglobulin gene DNA and exons are indicated by boxes. The S. aureus nuclease coding region is hatched. The locations of the heavy chain locus transcription enhancer element (E) and the gpt gene are indicated. Restriction endonuclease cleavage sites are abbreviated R, EcoRI; Xh, XhoI; S, SacI and Sl/Xh, a sequence formed by joining a SalI to an XhoI site. The sequence is presented around the XhoI site of plasmid pSV-$V_{NP}\gamma$SNase, which forms the junction of the γ2b CH2 exon and the SNase gene.

b) Diagrammatic representation of the predicted structures of the anti-NP antibodies whose heavy chains are encoded by the plasmids depicted in (a). Disulphide linkages (-S-) between heavy (H) and light (λ) chains are indicated. Only one bivalent subunit of the decavalent pSV-Vμ1 encoded IgM is illustrated..

Methods: The construction of pSV-Vμ1 has been detailed previously[5]. In all three plasmids, the V_{NP} exons are contained on a common EcoRI fragment and the vector is the BamHI - EcoRI fragment of pSV2gpt (ref.. 12) with an XhoI adapter in the BamHI site. pSV-$V_{NP}\gamma\delta$ contains an EcoRI - SacI and pSV-$V_{NP}\gamma$SNase an EcoRI - XhoI mouse Cγ2b fragment derived from phage λMYG9 (ref.. 13). Thus pSV-$V_{NP}\gamma\delta$ contains the γ2b CH1 and hinge exons whereas pSV-$V_{NP}\gamma$SNase also contains the 5'-end of the CH2 exon. The mouse $C\delta_g$ exon of pSV-$V_{NP}\gamma\delta$ is contained in a BamHI fragment of phage Ch257.3 (ref.. 14) which was obtained as a SacI - SalI fragment after cloning in M13mp11. The SNase coding region derives from an M13mp8 clone containing a S. aureus Sau3A fragment in the BamHI site (R.O.F., unpublished). Removal of the SNase gene from M13mp8 as a BamHI - SalI fragment and recloning in

M13mp12W (ref. 15) allowed its isolation as a XhoI - SalI fragment for final assembly of pSV-$V_{NP}\gamma$SNase.

Fig.2. SDS/polyacrylamide gel electrophoresis of anti-NP antibodies secreted by J558L transfectants. (a) Purified antibody (30 µg) that has been boiled with 2-mercaptoethanol prior to electrophoresis and stained with Coomassie blue (b) Biosynthetically labelled antibody analyzed after reduction. Samples purified from cells labelled in the presence of tunicamycin are marked Tm. (c) Unreduced antibody (30 µg) purified from culture supernatants.

Methods: J558L cells stably transfected with the pSV plasmids were obtained by spheroplast fusion and growth in selective medium essentially as described previously[5] except that HAT was omitted from the selective medium and mycophenolic acid was used at 5 µg/ml, as described by Oi et al[4]. Cells were cloned by limiting dilution. Antibody samples were purified from supernatants of cloned J558L transfectants grown in Dulbecco's modified Eagle's medium containing 5% foetal calf serum. Supernatants (2 litres) were passed over 2 ml columns of 4-hydroxy-5-iodo-3-nitrophenacetyl-aminocaproic Sepharose (NIPcap-Sepharose) and antibody eluted from the washed sorbent with 1 mM NIPcapOH in phosphate buffered saline. Biosynthetically labelled antibody was purified on 40 µl NIPcap-Sepharose columns from supernatants of cells incubated for 4 h at 37°C in medium containing L-[^{35}S]-methionine. Tunicamycin was, if required, included during the labelling and during a 2 h preincubation at 8 µg/ml; parallel incubations with an IgE secreting cell line confirmed the efficacy of the drug. Reduced samples were

analyzed on 12% polyacrylamide gels whilst 7% gels were used for unreduced samples.

Fig.3. Assay for nuclease activity. Single stranded M13 DNA (2 µg) was incubated at 37°C for 30 min in 25 mM sodium borate, 250 mM NaCl, 10 mM $CaCl_2$, pH8.5 (20µl) containing various amounts of $V_{NP}\gamma\delta$ or $V_{NP}\gamma$SNase antibody or of purified S. aureus nuclease. The quantities of antibody/enzyme used are given in nanograms. DNA in the samples was then analyzed by ethidium bromide fluorescence after electrophoresis through a 1.2% agarose gel. A HindIII digest of phage λ DNA provides size markers. Ca^{++} dependency of the nuclease activity was confirmed by running incubations in the presence of 40 mM $MgCl_2$, 25 mM EGTA.

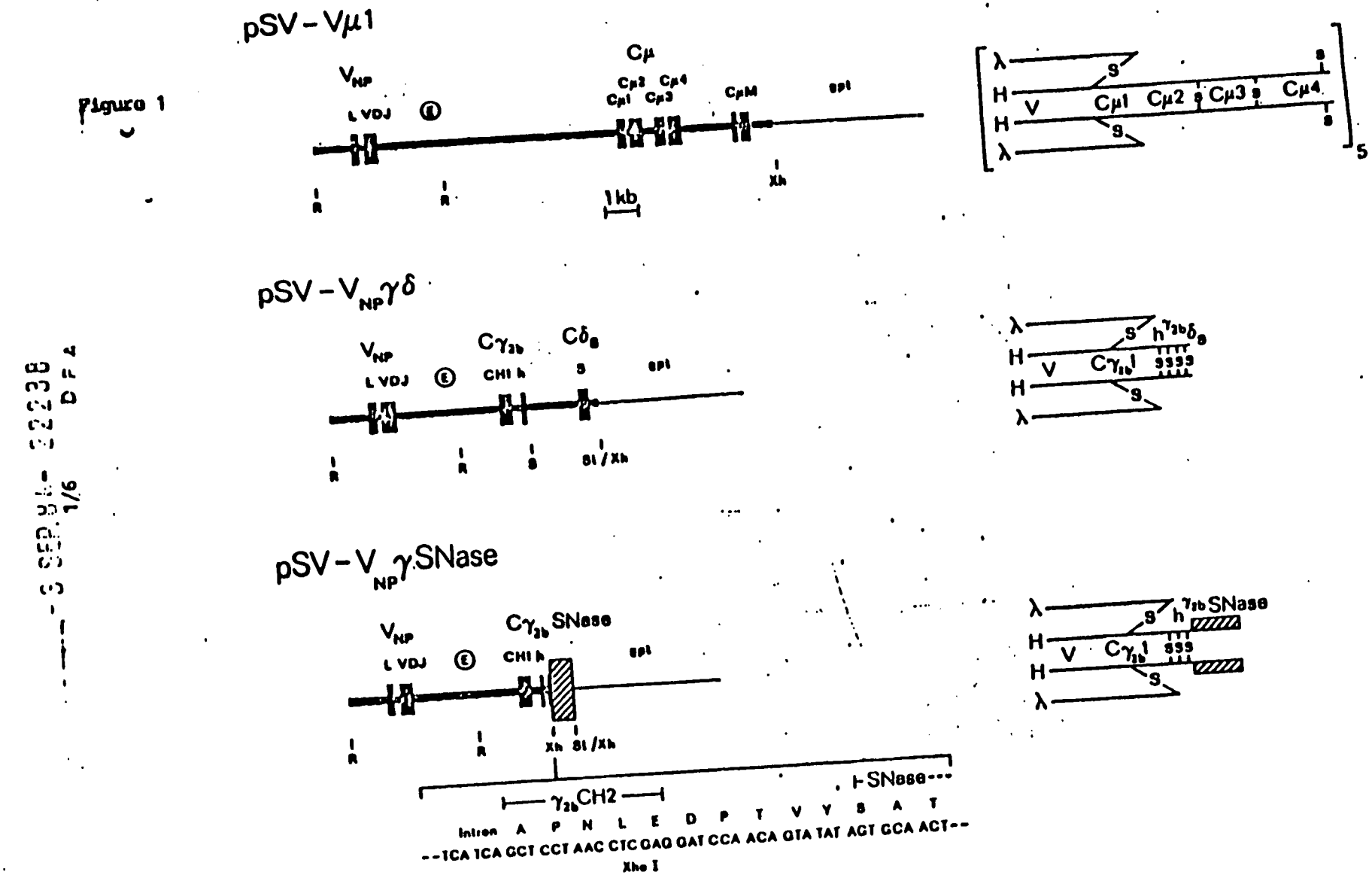
Fig.4 Use of $V_{NP}\gamma$SNase antibody in ELISA-type assay. Polyvinyl microtitre plates were coated with $(NIP)_{20}$-bovine serum albumin (40 µg/ml). After blocking unreacted sites with BSA, dilutions of $V_{NP}\gamma$SNase or $V_{NP}\gamma\delta$ antibodies were incubated in the wells; the amount of antibody - in nanograms - incubated in each well is indicated. After washing off unbound material, a solution (40 µl) containing 1 ug M13 single strand DNA and 1 µg/ml ethidium bromide in pH8.5 buffer (see Fig. 3 legend) was added. The plate was photographed after a 1 h incubation at 37°C.

Fig. 5 (a) Structure of pSV-$V_{NP}\gamma$=yc (b) Analysis of affinity purified $V_{NP}\gamma$myc on 10% SDS/polyacrylamide gel. The protein was reduced prior to electrophoresis and stained subsequently with Coomassie Blue. Methods: The structure of pSV-$V_{NP}\gamma$myc is identical to that of pSV-$V_{NP}\gamma\delta$ except that the pSV-$V_{NP}\gamma\delta$ SacI-XhoI fragment containing the $C\delta_s$ exon has been replaced by a SacI-BglII fragment containing the 3' exon of mouse c-myc. The

restriction site marked Bg/B is a site formed by joining the Bcl II site at the 3' end of c-myc to the BamHI site of pSV2gpt. The c-myc fragment comes from phage λMYC2, which contains the translocated c-myc gene of mouse plasmacytoma X63Ag8 (ref. 13).

Fig. 6 Assay for c-myc antigenic determinant in Fab-myc. Monoclonal anti-c-myc antibody CT14-G4.3 (gift of G. Evan) was coated (10 μg/ml) onto polyvinyl microtitre plates and unoccupied sites then blocked with BSA. Varying concentrations of either anti-NP Fab-myc ( ) or $F(ab)'_2$ ( ) were incubated in the wells and, after washing, bound anti-NP antibody was detected by developing with radioiodinated monoclonal anti-idiotope antibody Ac38. The Ac38 antibody (gift of M. Reth & K. Rajewsky) recognizes the variable region of the anti-NP antibodies[16]. Controls performed with Ac38-reactive monoclonal IgM, IgD and IgD antibodies (100 μg/ml) gave less than 400 cpm.

Figure 1


pSV - Vμ1
Cμ
V_{NP}
L VDJ
E
Cμ2 Cμ4
Cμ1 Cμ3
CμM
spl
Xh
R
R
1kb
λ
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V
Cμ1 Cμ2 S Cμ3 S Cμ4
S
5
pSV - V_{NP}γδ
$Cγ_{2b}$
$Cδ_S$
CH1 h
S
Sl / Xh
$h^{γ_{2b}δ_S}$
$Cγ_{1b}$ I
SSSS
pSV - V_{NP}γSNase
$Cγ_{2b}$SNase
$γ_{1b}$SNase
SNase---
$γ_{2b}$CH2
Intron A P N L E D P T V Y S A T
--TCA TCA GCT CCT AAC CTC GAG GAT CCA ACA GTA TAT AGT GCA ACT--
Xho I

Figure 3

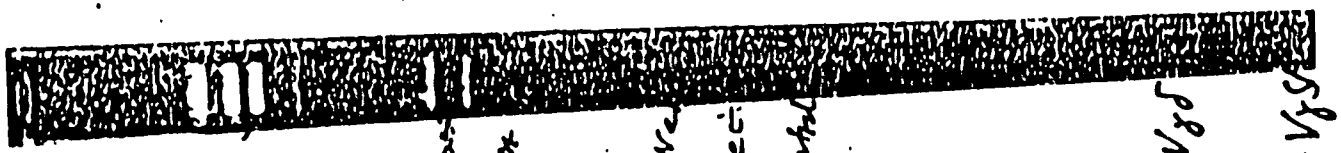
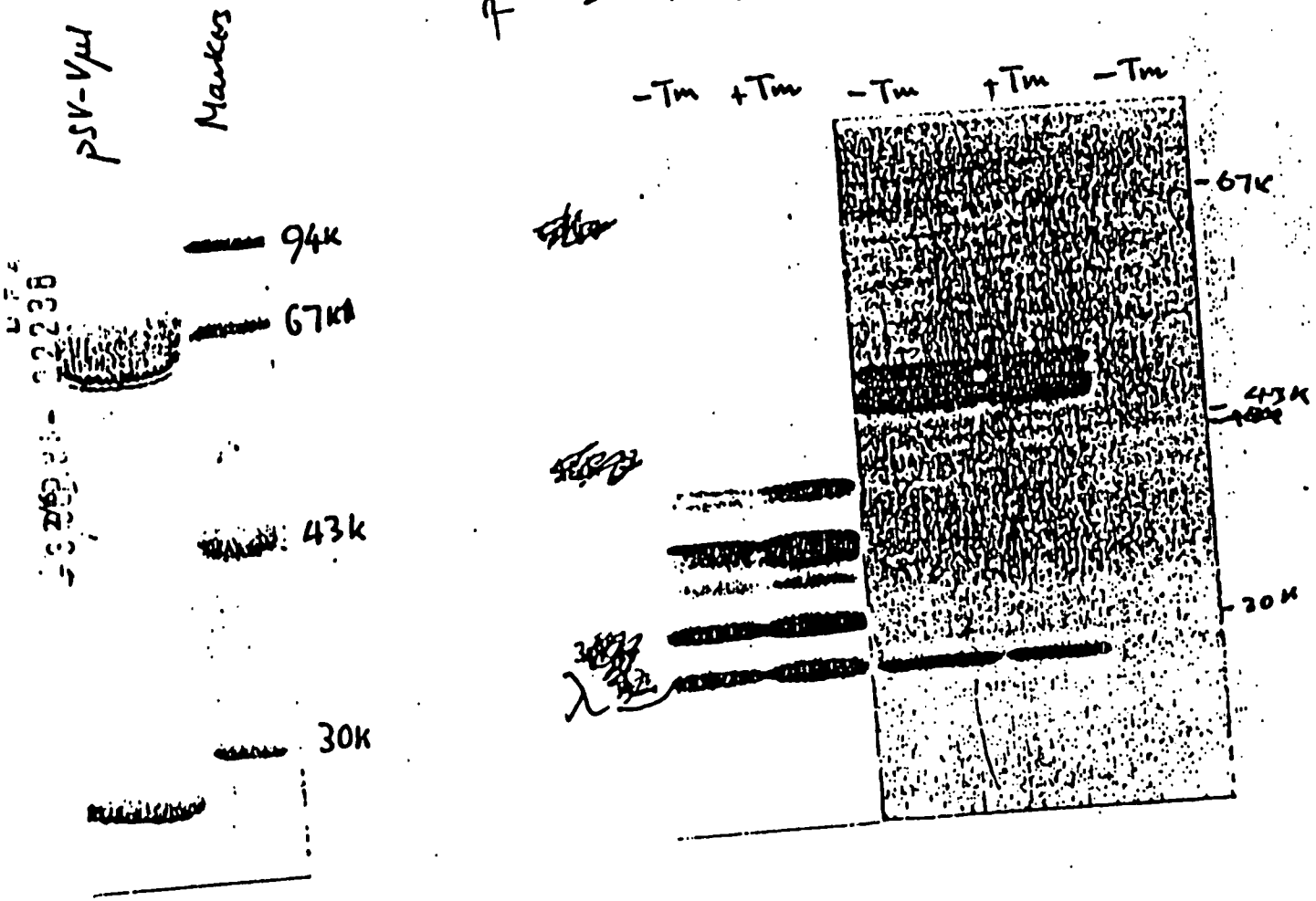


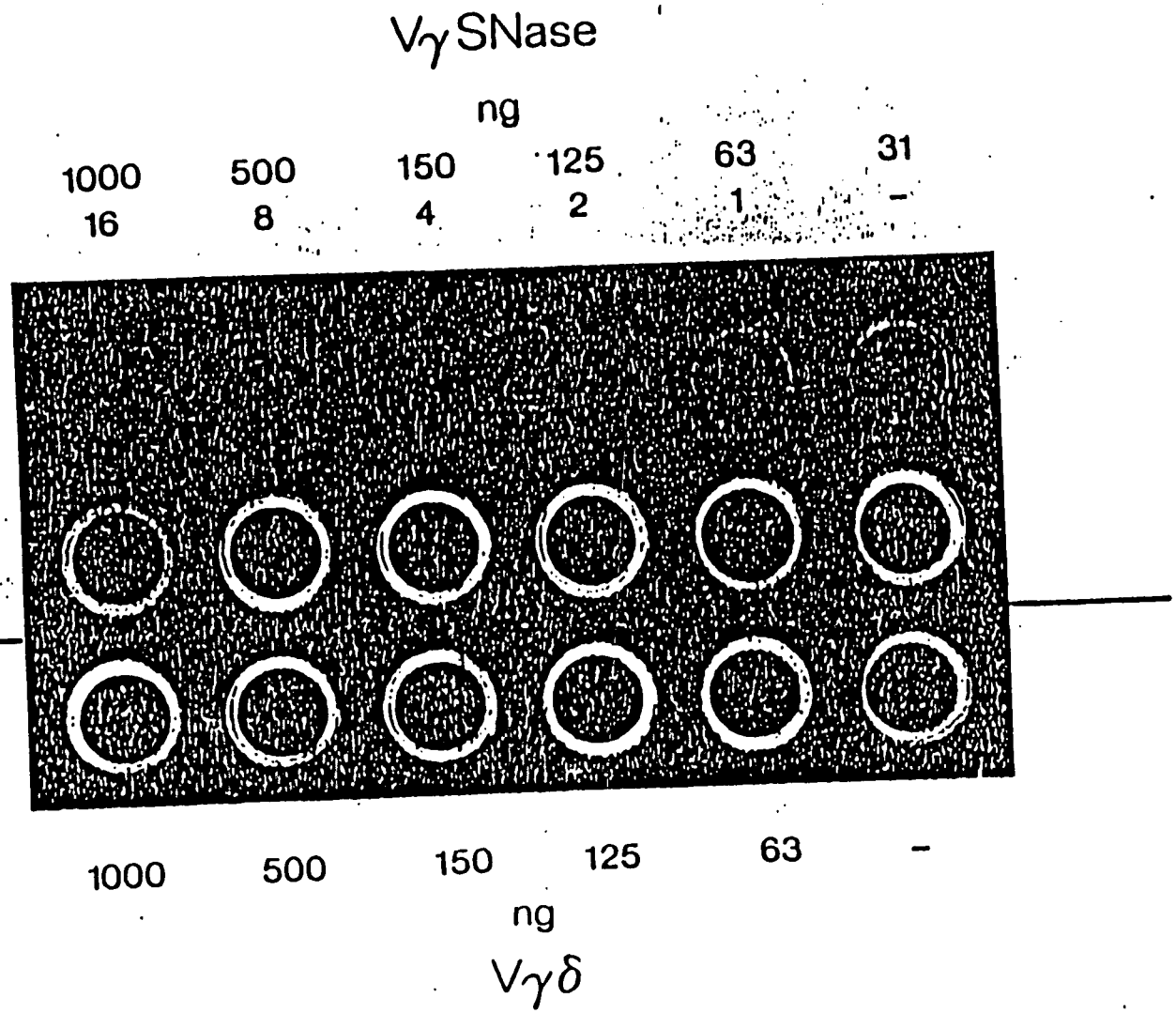
Figure 2



Marker
Vγδ
200
400
80
16
VγSNase
2000
400
80
16
SNase
160
16
1·6
0·16
– VγSNase
2000
SNase
160
Marker

Figure 3

Figure 4



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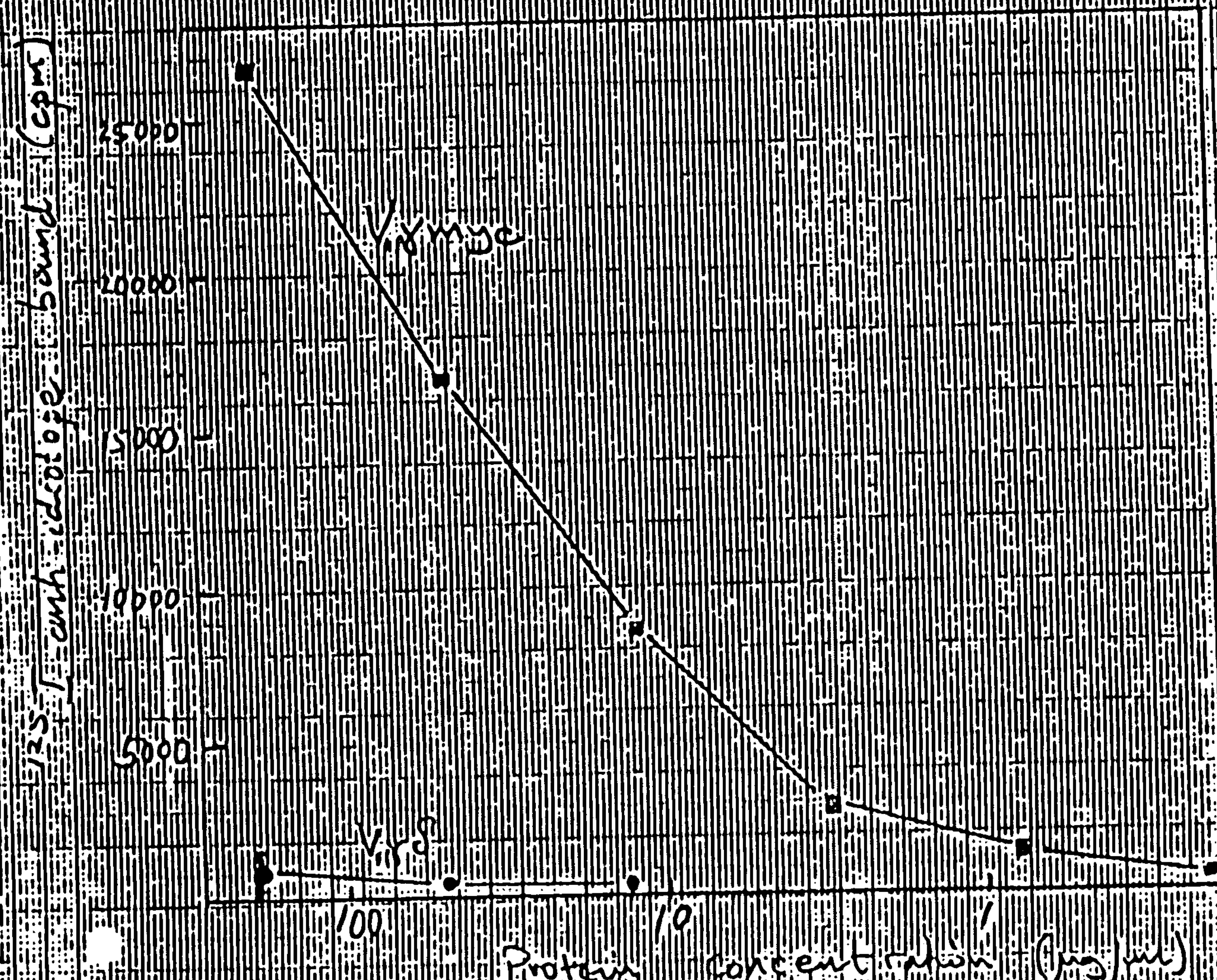
125I anti-idiotope bound (cpm)
25000
20000
15000
10000
5000
100
10
1
Protein concentration (µg/ml)

Figure 6

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